

ADP Hunter™ Assay for HTS of Kinase Inhibitors using the PHERAstar

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- Generic screening of potentially all protein kinases in fluorescence mode using the PHERAstar
- Direct measurement of ADP and therefore cost effective: No need of specific antibodies or radioactive beads
- Simple and robust assay with $Z' > 0.7$

Introduction

As mediators of eukaryotic signal transduction, controlling multiple cellular processes such as gene transcription, cell cycling, migration, apoptosis and differentiation, protein kinases are considered important targets in drug discovery. Kinase HTS screens ideally require kinase assay platforms with broad applicability in the inhibitor drug discovery process and are thus generically applicable to both the kinase target and substrate. Most non-radioactive kinase assay formats are limited in this respect in that they require either specific antibodies, or affinity capture reagents, to detect generation of the phosphorylated substrate. Often specific antibodies to the phosphorylated substrate are unavailable while affinity capture methods have limitations in the kinase assay conditions that can be used. During substrate phosphorylation, all kinases consume ATP and assays have been developed to measure ATP depletion occurring during the kinase reaction. However, this approach is limited by the ATP concentration required by the kinase, resulting in a high assay background. Consequently, small signal decreases, as occurs with weakly active kinases are difficult to detect.

An optimal approach is to measure the accumulation of a generic product of the kinase reaction i.e. ADP. This technique results in an increase in assay signal directly proportional to kinase activity and has the marked advantage that the assay is performed at a range of ATP concentrations, including those at the ATP K_m value. Consequently, the inhibitory potency of novel compounds, when evaluated at the ATP K_m , is a robust measure of activity at the ATP binding site and is easily compared to similar measurements at other kinases. Moreover, measurement of ADP accumulation allows flexibility in choice of the kinase substrate, so that both peptides and endogenous substrates (including during autophosphorylation) the kinase may be utilized.

DiscoverRx has developed a homogeneous fluorescence based assay to measure the generation of ADP, a universal product of kinase activity. The assay uses an enzyme-coupled reaction that produces a red-shifted fluorescence signal that is directly proportional to the amount of ADP in the solution.

ADP Hunter is a biochemical assay to measure the accumulation of ADP (figure 1), a universal product of kinase enzyme activity.

Step 1: Kinase Reaction



Step 2: ADP Detection

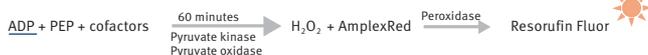


Fig. 1: Schematic principle of the ADP Hunter assay

ADP Hunter is specifically designed for high throughput screening of kinase inhibitors. The assay has been designed for use in full-volume 384-well microplates, but can also be run in additional microplate formats. To allow for automation, a Stop Solution is also provided for added signal and background stabilization.

Unlike alternative generic approaches that monitor the depletion of ATP from a kinase reaction, this method follows the product of the reaction, and offers a convenient gain-of-signal assay format. This kinase assay is a generic, non-radioactive method that doesn't require use of antibody. This application note describes the use of the ADP Hunter assay measured on BMG LABTECH's PHERAstar multimode HTS plate reader.

Materials and Methods

BMG LABTECH's PHERAstar combines rapid plate reading necessary for HTS with the enhanced performance and sensitivity needed to read small fluid volumes (figure 2).



Fig. 2: BMG LABTECH's high-end multimode plate reader PHERAstar was used to perform the ADP Hunter kit.

The new multimode microplate reader has the flexibility to excel with the most demanding assays and is designed to read all leading HTS detection modes such as fluorescence intensity, time-resolved fluorescence, fluorescence polarization, luminescence and absorption in all formats up to 1536. The PHERAstar was run in fluorescence mode for the monitoring of the ADP Hunter demo kit (DiscoverRx Corporation, cat #90-0077) containing the following reagents:

Table 1: ADP Hunter demo kit components.

Kit Components		Volumes
1	ADP Hunter Reagent A	8 mL
2	ADP Hunter Reagent B	15 mL
3	ADP Hunter Stop Solution	4 mL
4	ADP Hunter Standard (360 μM)	2 mL
5	ADP Hunter Assay Buffer	20 mL

The standard curve for DiscoverRx's ADP Hunter kit was run according to the package insert protocol (table 2) in black walled 384-well plates (non-binding polypropylene plates; Greiner cat# 781209).

Table 2: ADP Hunter protocol for a standard curve

Full Volume 384-well Plate	ADP Standard
Step 1: Standard Dilutions	8 μ L ADP Standard dilutions
Step 2: ADP Detection	a) Add 8 μ L Reagent A b) Add 16 μ L Reagent B c) Incubate 60 minutes
Step 3: Stop Solution	Add 4 μ L Stop Solution
Read fluorescence intensity signal: Resorufin excitation wavelength: 530-10 nm Resorufin emission wavelength: 590-20 nm	
Note: The signal may be measured up to 2 hours after addition of Stop Solution	

The fluorescence signal (Ex: 530-10 nm / Em: 590-20) was read 1 hour after the addition of the last reagent on the BMG PHERAstar using the following protocol for plate reader setup (figure 3).

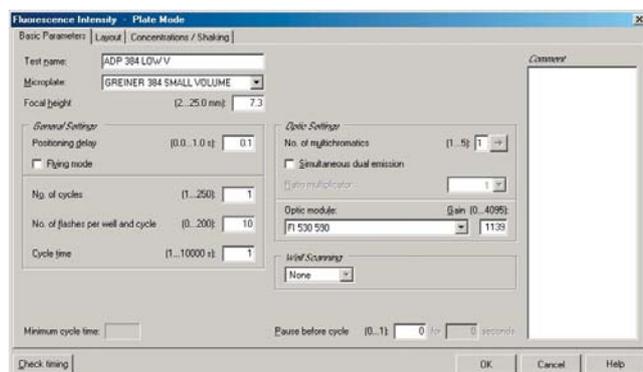


Fig. 3: This screenshot shows the ADP Hunter assay setup window from the PHERAstar, an optical module for the ADP Hunter is directly available from BMG LABTECH.

Results and Discussion

DiscoverX ADP Hunter assay was prepared in 384-well format and standard curves were run on BMG LABTECH's PHERAstar in fluorescence mode. ADP standard detection reagents were added according to the assay protocol and the fluorescence signal was read 1 hour after addition of the last reagent. The ADP standard curve is illustrated in the figure below (figure 4).

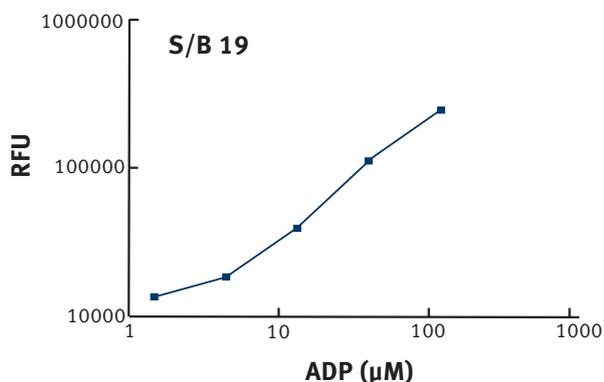


Fig. 4: ADP Hunter standard curve data run in 384-well format

The ADP Hunter assay format was designed to produce a positive signal in direct proportion to the amount of ADP generated. The dynamic range of the assay is 1.5 μ M to 120 μ M ADP. The simple homogeneous assay format, with a single sixty minute incubation, provides robust and reproducible results with signal to background ratios > 10 and typical Z' values > 0.7 .

A series of product features make ADP Hunter the ideal screening solution, while maintaining an assay format that requires no waiting for or testing of antibodies, tolerates a broad range of ATP concentrations, and uses unmodified substrates or whole proteins.

ADP Hunter is based on enzyme coupled reaction that produces a positive red-shifted fluorescence signal, minimizing interference from fluorescent compounds. In addition, ADP Hunter incorporates a stop solution, which stabilizes the detection signal for screening plates in batch mode.

Here we have described the value of a generic kinase screening assay when analyzed on a PHERAstar instrument. Together, they offer a powerful solution for kinase screening needs.

Conclusion

This novel technology recently developed and commercialized by DiscoverX Corp provides generic approaches to kinases research and inhibitor. This assay is a useful tool for those customers who do not have access to a modified substrate, a phosphorylation-state specific antibody, or the ability or desire to use radioactivity. These reagents are designed to be robust and applicable to high throughput fluid dispensing systems using simple plate readers. The PHERAstar with its innovative and user friendly, intuitive software allows for the greatest flexibility and ease of use.

For more information on DiscoverX assays please refer to the web site:

www.discoverx.com

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